JHR 98: 1133–1152 (2025)
doi: 10.3897/jhr.98.150721
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Differential oviposition timing by seasonality and oviposition timing of parasitoids of the invasive roseau cane scale (*Nipponaclerda biwakoensis*) as revealed by multiplex PCR assays

Violet Sparks[1], Ilgoo Kang[2], Morgan Carrere[1], Hannah Broadley[3], Rodrigo Diaz[1]

1 *Department of Entomology, 404 Life Sciences, Louisiana State University, Baton Rouge, LA 70803 USA* **2** *Department of Entomology, Kyungpook National University, 2559 Gyeongsang-daero, Sangju, Gyeongsangbuk-do 37224 Republic of Korea* **3** *Forest Pest Methods Laboratory, USDA APHIS PPQ S&T, 1398 West Truck Road, Buzzards Bay, MA 02542 USA*

Corresponding author: Violet Sparks (tspark3@lsu.edu)

Academic editor: Petr Janšta | Received 18 February 2025 | Accepted 1 December 2025 | Published 17 December 2025

https://zoobank.org/69831A5D-574D-48ED-ACF2-215F68C278BF

Citation: Sparks V, Kang I, Carrere M, Broadley H, Diaz R (2025) Differential oviposition timing by seasonality and oviposition timing of parasitoids of the invasive roseau cane scale (*Nipponaclerda biwakoensis*) as revealed by multiplex PCR assays. Journal of Hymenoptera Research 98: 1133–1152. https://doi.org/10.3897/jhr.98.150721

Abstract

Roseau cane scale, *Nipponaclerda biwakoensis* (Hemiptera: Aclerdidae) is an invasive species negatively impacting *Phragmites australis* (Cyperales: Poaceae) stands within the Mississippi River Delta. Previous research generated a PCR multiplex capable of identifying the three species of parasitoid wasps (Hymenoptera: Encyrtidae) that attack the scale in its invasive range. The appearance of a fourth species, *Aprostocetus* sp. (Hymenoptera: Eulophidae), within Louisiana in 2022 necessitated updating the multiplex with primers specific to the new species. The newly updated multiplex can identify the presence of all four parasitoid species within their host. DNA was extracted from the whole bodies of scales collected at different life stages and three different dates during the growing season and PCR performed with the new multiplex. Our findings show each species within the complex oviposits within a specific window of nymphal development which might help explain how successful multiparasitism frequently occurs in this system. The frequency of multiparasitism and relative abundance of each parasitoid species was consistent across the three sampling dates, except *Astymachus lasallei* (Hymenoptera: Encyrtidae), which was found in higher abundance during the end of the growing season.

Keywords

Competition, genetic identification, multiparasitism, oviposition, *Phragmites*, roseau cane scale, temporal niche partitioning

Copyright: This is an open access article distributed under the terms of the CC0 Public Domain Dedication.

Introduction

Roseau cane scale, *Nipponaclerda biwakoensis* (Kuwana) (Hemiptera: Aclerdidae) (hereafter referred as RCS), is invasive to the Gulf Coast, United States where it feeds exclusively on *Phragmites australis* (Cav.) Trin. Ex Steud. It is native to east Asia in the countries of China, Japan, South Korea, and Taiwan and has been collected as far as Beijing, China in the north and Hong Kong in the south (Cortez et al. 2022; Schneider et al. 2022). Scales aggregate under the leaf sheathes of host plants where they feed on the sap from vascular tissues. Mature females are approximately 5 mm long, brown, and give birth (ovoviparity) between 300 and 600 1st instar nymphs (crawlers) at the end of their lives (Kaneko 1995; Lopez 2022). Crawlers are the most mobile of the life stages, complete with six legs, eyes, and antennae (McConnell 1953) (Fig. 1A) and disperse from under the mother scale in search of appropriate feeding locations along the stem. Once they begin feeding, the immature scale molts, losing its appendages and eyes, and begins producing wax around the cuticular margin (McConell 1953; Kaneko 1995). Males of the species are less than 2 mm, apterous, and have simple legs

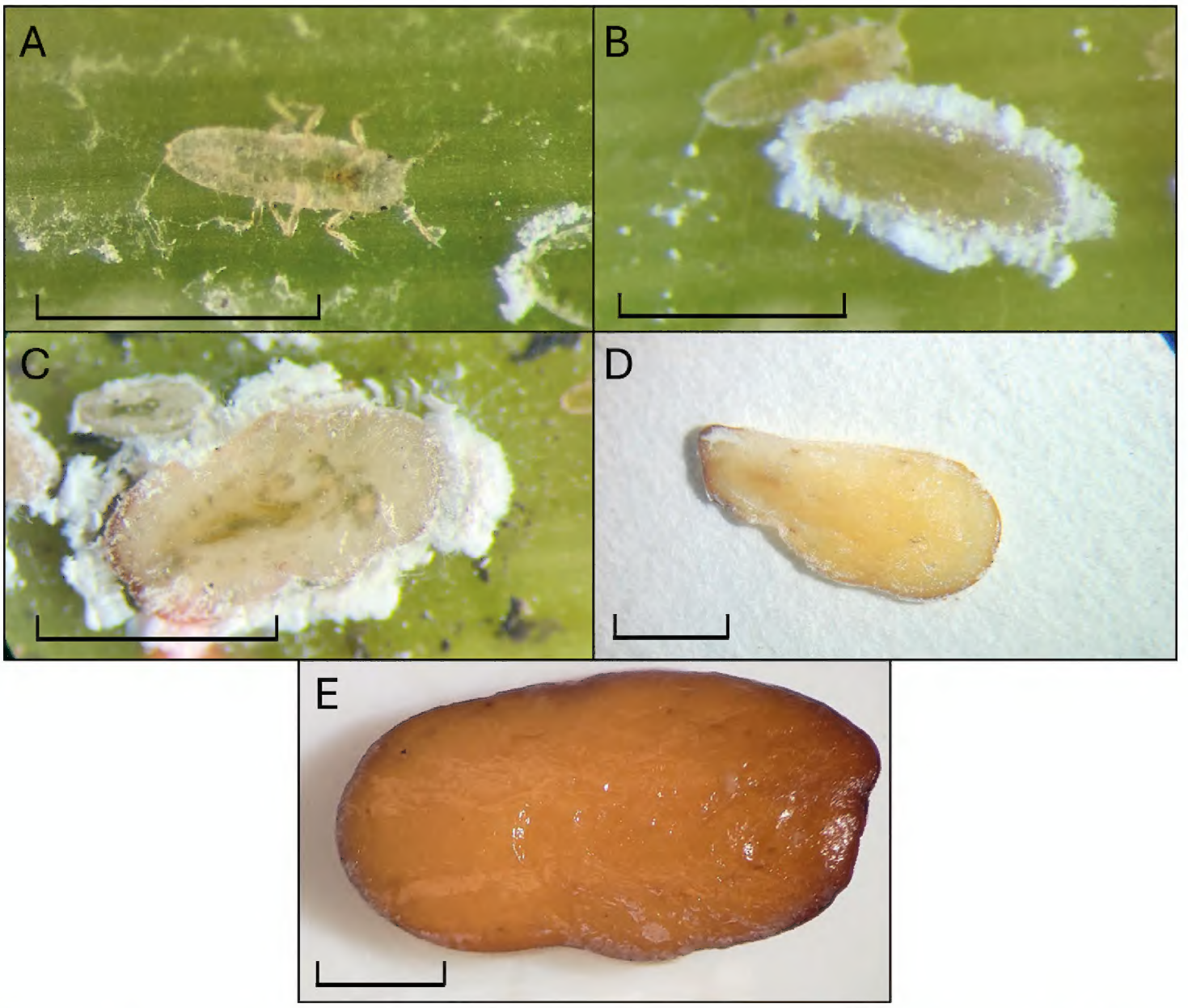

Figure 1. Roseau cane scale development stages. **A** 1st instar (crawler) **B** 2nd instar **C** 3rd instar **D** 4th instar **E** mature female. Scale bars: 0.5 mm (**A, B**); 1.0 mm (**C, D, E**).

they use to move towards nearby females with which they mate (McConell 1953). The life cycle takes between one and two months to complete with three to six overlapping generations each growing season, with faster development times and more generations associated with warmer climates like Taiwan (Kaneko 1995, 2004; Cortez et al. 2022).

RCS was first reported outside its native range on *Phragmites* stands in the Lower Mississippi River Delta, Plaquemines Parish, Louisiana, United States in 2017 (hereafter referred to as MRD). Widespread die-back of *Phragmites* stands in the MRD (see Suppl. material 1) were associated with RCS populations as high as 2,000 scales per stem, suggesting that these outbreaks could be a major plant stressor (Knight et al. 2018). Given that *Phragmites* is the dominant vegetation within the MRD, this die-back raised concerns about potential ecological harm and wetland loss (Ramsey and Rangoonwala 2017; Cronin et al. 2020; Knight et al. 2020). Management of RCS within the MRD faces several challenges that make typical pest management tools ineffective. Insecticides are a not feasible tool, as the scales are difficult to spray due to their location underneath the leaf sheathes, and their use could endanger non-target wildlife inhabiting *Phragmites* stands. Prescribed burns, as used in cultivated *Phragmites* in China (Brix et al. 2014), risks damaging the numerous oil pipelines and pumping stations present in the MRD (Suppl. material 2). This leaves biological control of RCS as the most suitable option to achieve management.

Initial investigations into RCS parasitism found three species present in Louisiana: *Boucekiella depressa* Hoffer, *Neastymachus japonicus* Tachikawa, and *Astymachus lasallei* Noyes and Higashiura (Hymenoptera: Encyrtidae) (Fig. 2A, B, D) (Knight et al. 2018; Cortez et al. 2022; Pruett et al. 2025). These three are found throughout the RCS native range and are thought to have arrived in Louisiana alongside their host (Kaneko 1995; Knight 2018; Cortez et al. 2022; Pruett et al. 2025). Species within the complex are noted as being gregarious (multiple individuals develop from the same host) endoparasitoids that consume all but the exocuticle of their host over the course of their development, with adults exiting from adult female mummies (Kaneko 1995). *Astymachus lasallei* and *N. japonicus* are known only to attack RCS while *B. depressa* has been collected from multiple hosts (Kaneko 1995; Herting 1997; Noyes and Higashiura 2020). In the fall of 2022 collections of parasitized RCS in Louisiana reared a species of *Aprostocetus* Westwood (Hymenoptera: Eulophidae) (Fig. 2C) that was morphologically similar to those found in the native range (Kaneko 1995, 2004, 2005a, b; Cortez et al. 2022) and was previously unreported in the region. Within a year of its discovery in Louisiana, *Aprostocetus* sp. was found in nearly every *Phragmites* patch infested by RCS in the adventive range (V. Sparks unpublished data).

Previous work with RCS parasitoids has relied on the rearing of adult wasps from isolated hosts to make species level identification (Kaneko 1995, 2004, 2005a, b; Knight et al. 2018; Cortez et al. 2022). The larvae of these parasitoids are morphologically indistinct, and it cannot be guaranteed that all larvae within the host are of the same species due to the frequency of multiparasitism. Rearing adult wasps is not a perfect method either as it takes time for parasitoids to emerge, only parasitoids in the later stages of development may survive having their host removed from the stem, and infor-

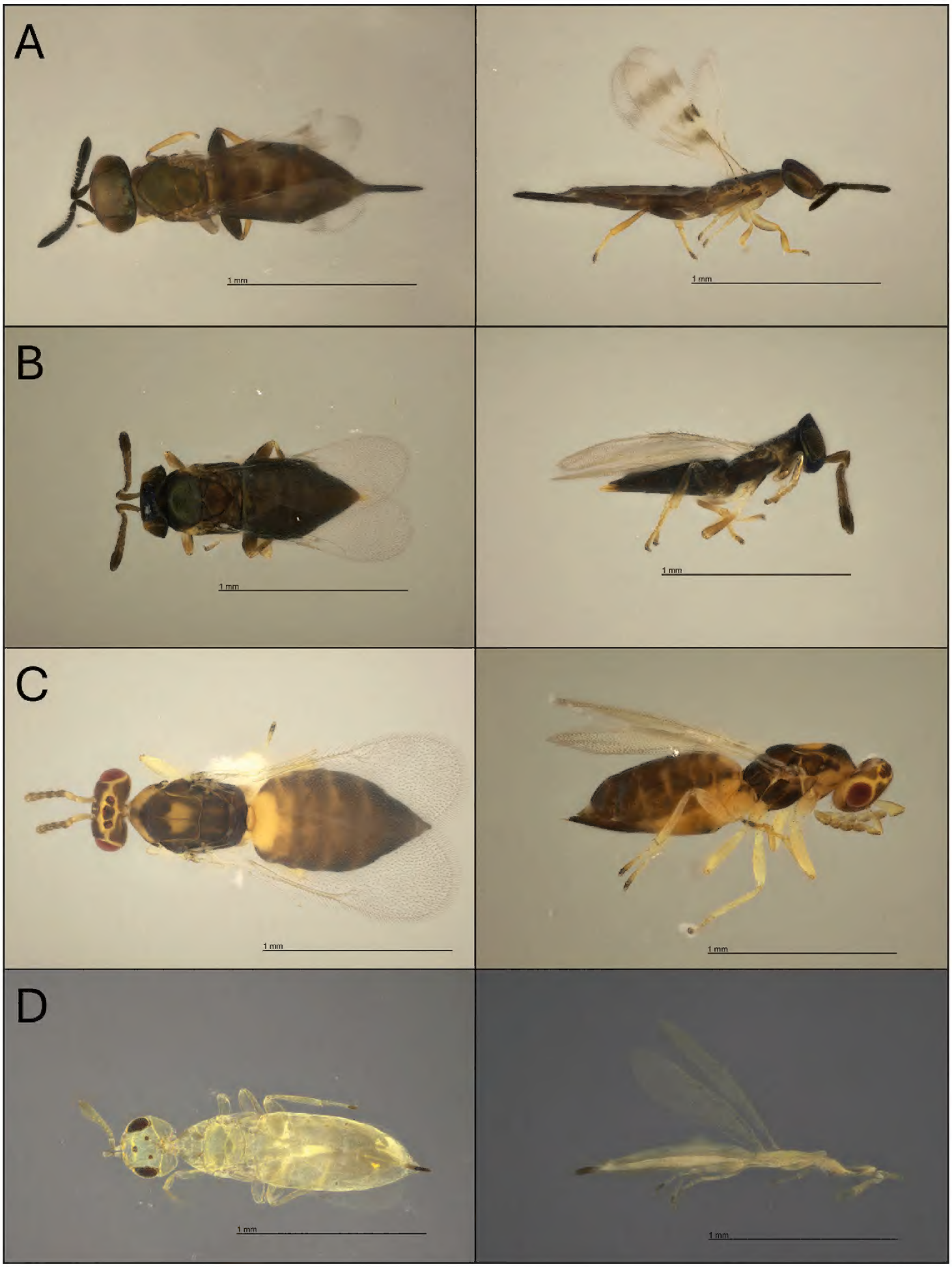

Figure 2. Dorsal (left) and lateral (right) view of **A** *Boucekiella depressa* **B** *Neastymachus japonicus* **C** *Aprostocetus* sp. **D** *Astymachus lasallei*. Scale bars: 1.0 mm.

mation is lost for those that fail to complete development (Agusti et al. 2005). Polymerase chain reaction (PCR)-based molecular identification techniques are frequently used in parasitoid research due to their ability to accurately identify a species regardless of life

stage and can differentiate between morphologically similar cryptic species (Smith et al. 2006, 2008; Gariepy et al. 2007). Diagnostic PCR-based assays are able to detect even small amounts of parasitoid DNA within a host, including eggs and early instars, because they can be designed to bind and amplify to specific DNA sequences (Tilman et al. 2000; Greenstone 2006). Combining multiple species-specific primers into a viable multiplex allows for single-step identification of parasitoids within a host in systems where multiple parasitoid species may be expected, and in the case of RCS, multiple species present within the same host (Traugott et al. 2006; Pruett et al. 2025).

One of the outstanding aspects of the RCS parasitoid complex is the frequency with which successful multiparasitism, two or more species completing development from the same host, occurs. All combination pairs of each species and as many as three species have been observed emerging from the same host (Kaneko 1995; Cortez et al. 2022; Pruett et al. 2025; V. Sparks pers. obs.). Successful multiparasitism is supposed to be a rare phenomenon in Hymenoptera (Miller 1982). The larvae of solitary parasitoids typically come equipped with large mandibles and are highly mobile, allowing them to kill and consume other competitors within the host (Harvey et al. 2009). In typical intrinsic competition scenarios, there is an inferior and a superior competitor that is more likely to win the competition, but the inferior competitor can reclaim the advantage by ovipositing and developing ahead of the superior species (Hood et al. 2021). However, gregarious parasitoids, like the RCS parasitoids, lack these weapons and instead cohabitate with the other larvae of the same species (superparasitism) inside the host, whom are most likely also their siblings (Godwin and Odell 1984) or with the larvae of other parasitoid species in cases of multiparasitism.

The first descriptions of the RCS parasitoid complex by Kaneko (1995) claim oviposition by all species occurs within immature females in their latest stage of development; however, it was not stated how this was concluded (such as first appearance of parasitoid larvae, dissection for eggs, or direct observation of oviposition behavior). The simultaneous oviposition by multiple species, if true, could explain why multiparasitism frequently occurs within the system as no one species has a head start on development over the others (Kaneko 1995). It is necessary in a biological control program to identify when the pest is vulnerable to attack by its natural enemies and so additional research on multiparasitism and oviposition timing in the RCS parasitoid community in the adventive range is needed. As Abell et al. (2020) indicates, releases performed at inopportune times may see a loss of agent effectiveness simply because populations of the target pest are not currently at a life stage the natural enemy can utilize.

Previous work by Pruett et al. (2025) successfully produced species-specific PCR primers that were then combined into a multiplex for the molecular identification of *B. depressa*, *N. japonicus*, and *A. lasallei* DNA within RCS. With the arrival of *Aprostocetus* sp. to Louisiana, the multiplex needs to be updated to allow for the identification of all RCS parasitoids currently within the adventive range. The objectives of this study were to (1) develop species-specific PCR primers for *Aprostocetus* sp., (2) incorporate the newly developed primers into the already existing encyrtid multiplex, and (3) determine the host life stage window each species parasitizes their host by performing PCR with the multiplex on different RCS life stages.

Methods

Collection of scales and parasitoid wasps

RCS were collected from field sites within the MRD, Plaquemines Parish, Louisiana, USA in October 2022 (Fall), May 2023 (Spring), and August 2023 (Summer). Roseau cane stems were haphazardly selected, cut at the soil or water line, and transported back to Louisiana State University, Baton Rouge, Louisiana where leaf sheathes were removed to expose the scales underneath. Living scales were collected and preserved in ethanol at 4 °C for later DNA extraction. Collected scales were measured across their longest point using 10 mm microscope rulers under a National DC3-420T digital microscope (National Optical, Schertz, TX, USA) and sorted into 1st instar larvae, 2nd through 4th instar larvae, and mature males and females (Table 1, Fig. 1). Size ranges for the 2nd through 4th instars were arbitrarily created based on a general trend of observing three size classes of immobile larvae (small, medium, and large). Visual indications (eggs, larvae, pupae present) of current parasitism were noted for each scale specimen. Parasitized mature scales with fully developed larvae were placed into gel capsules and adult wasps reared for morphological identification instead of storing in ethanol. Reared adult wasps were used for positive control DNA and for development of species-specific primers. Images of scale life stages and parasitoids were taken using the Visionary Digital BK Plus imaging system (Dun, Inc.) equipped with a Canon® EOS 5DS DSLR and were stacked using Zerene Stacker™ v. 1.04 (Zerene Systems LLC.). Adobe Photoshop® CS 6 was used to edit images.

DNA extraction

Genomic DNA was extracted from immature and adult scales as well as parasitoid wasps using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). The full body was ground using Fisherbrand™ Rnase-Free Disposable Pellet Pestles (Fisher Scientific, Carlsbad, CA, USA) in 180 µL of ATL buffer and 20 µL of proteinase K solution (Qiagen, Hilden, Germany). Samples were then incubated overnight at 56 °C. The remaining steps were followed according to the manufacturer's protocol, except the final elution step was performed twice using 100 µL AE buffer each time for a total of 200 µL final DNA product.

Table 1. Size and physical descriptions used to categorize life stages of the roseau cane scale, *Nipponaclerda biwakoensis*. Development is split into a mobile 1st instar nymph (crawler), three immobile nymphal instars (2nd-4th), and mature males and female.

Instar	Length (mm)	Physical Characters
1st	< 0.5	Legs present, no wax secretions
2nd	0.5-1.5	Loss of legs and antennae, wax secretion
3rd	1.5-3.0	Wax secretion buildup
4th	> 3.0	Lack of melanization
Mature (female)	> 3.0	Complete melanization
Mature (male)	< 2.0	Pink, legs present, cylindrical body

Aprostocetus sp. primer development and incorporation into multiplex PCR

Previous research with RCS parasitoids in Louisiana designed species-specific PCR primers for *Boucekiella depressa*, *Neastymachus japonicus*, and *Astymachus lasallei* that produce 155 bp, 221 bp, and 495 bp bands, respectively (Pruett et al. 2025). We designed species-specific forward primers that produce 375 and 303 bp DNA sequences of *Aprostocetus* sp. when amplified with the encyrtid reverse primer previously developed by Pruett et al. 2025 (Table 2).

To find appropriate forward primer regions, we downloaded 28S DNA sequences of *B. depressa* (656 bp, accession number: MN705818), *N. japonicus* (593 bp, accession number: MN705819), and *A. lasallei* (620 bp, accession number: MN705815) originally submitted to GenBank by K. Pruett. 28S sequences from *Aprostocetus* sp. were acquired from PCR using 28S-D2-3665F and 28S-D2-4068R universal primers (Hancock et al. 1988; Campbell et al. 1994) with DNA extracted from whole specimens. PCR products were then submitted to the LSU genomics facility (Baton Rouge, LA) and cleaned up using a primer depletion clean-up method developed by the facility. Purified products were bi-directionally sequenced on the 3130xl Genetic Analyzers (Applied Biosystems) using the BigDye Terminator v3.1 chemistry (Applied Biosystems). The obtained DNA sequences were edited and assembled using Geneious Prime 2021.2 (https://www.geneious.com) and compared with previously deposited parasitoid DNA sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

Sequences of 28S gene region of the four RCS parasitoids were aligned using the Multiple Sequence Alignment Viewer 1.23.0 (https://www.ncbi.nlm.nih.gov/projects/msaviewer/) and compared. We found two potential regions within the 28S gene sequence that could be used for designing forward species-specific primers for *Aprostocetus* sp. producing 375 and 303 bp DNA sequences when conducting PCR with the encyrtid reverse primer. Using the Oligo Analysis Tool provided on the website of Eurofins (https://eurofinsgenomics.com/en/resources/tools/oligo-analysis/), the properties of two potential forward primers were calculated, and the cross-dimer formation for each pair was evaluated. Analysis results indicated both forward primers could be used effectively in PCR.

Table 2. Primer information including gene region, name, and sequence for all PCR primers discussed in this project.

Gene	Primer name and direction	Sequence (5' → 3')	Source
28S	BD_F (F)	CAAGCCAGGCAAAAAATCG	Pruett et al. 2025
28S	NJ_F (F)	GACGAAACCGGTCCAACC	Pruett et al. 2025
28S	AL_F (F)	GACCAAAGACCGGAGCCA	Pruett et al. 2025
28S	Encyrtid_28S_R (R)	CACGAGACCGATAGCGAACA	Pruett et al. 2025
28S	28S-D2-3665F (F)	AGAGAGAGTTCAAGAGTACGTG	Hancock et al. 1988
28S	28S-D2-4068R (R)	TTGGTCCGTGTTTCAAGACGGG	Campbell et al. 1994
28S	Aprostocetus_F1 (F)	TTGCGGCCCATTACCGTGA	Newly developed
28S	Aprostocetus_F2 (F)	GCCCGGAGGCGCAAAC	Newly developed

The two newly designed primers were tested in singleplex with the encyrtid reverse primer and when incorporated into the encyrtid multiplex PCR using wasp DNA extracted both directly from wasps and from wasp immatures present in RCS samples.

Singleplex PCR amplification and gradient PCR

PCR amplification was run to confirm whether each species-specific primer amplifies DNA of their respective target organism when used with the Encyrtid_28S_R primer. Each 25 µl PCR amplification reaction volume contained 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 µl of DNA template, 9.5 µl of ddH2O, and 1 µl of the respective forward and reverse primer at 10 µM. PCR conditions were 94 °C for 3 minutes; 35 cycles of 94 °C for 30 seconds, either 52 °C for Aprostocetus_F1 or 57 °C for Aprostocetus_F2 and all other forward primers for 30 seconds and 72 °C for 1 minute; and a final extension at 72 °C for 5 minutes. To find out the optimal PCR annealing temperature of each primer set, we conducted gradient PCR using a PTC-200 thermal cycler (MJ Research, Massachusetts, USA). The gradient PCR condition was 94 °C for 3 minutes; 35 cycles of 94 °C for 30 seconds, 45–64 °C for 30 seconds and 72 °C for 1 minute; and a final extension at 72 °C for 7 minutes. Bands from all five forward primers were visible at 58 °C and so that temperature was selected as the annealing temperature for latter multiplex PCR.

Multiplex PCR

Multiplex PCR was performed using a T100 thermal cycler (Bio-Rad, Hercules, USA) with a total volume of 25 µl per reaction, containing 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 2.5 µl of primer mix, 1 µl of DNA template, and 8–9 µl of ddH2O. Optimization of primer concentrations was performed through trial and error testing of the multiplex with stock DNA extracted from adult parasitoids. The primer concentrations found to be most optimal for amplification of DNA products from all four species were: each forward primer for *B. depressa*, *N. japonicus*, and *A. lasallei* 0.096 µM and for *Aprostocetus* sp. was 0.064 µM, and the final concentration of the reverse primer was 0.35 µM (i.e., 50 µl of primer mix contains 1.2 µl of 100 µM forward primers for *B. depressa*, *N. japonicus*, and *A. lasallei*, 0.8 µl of 100 µM forward primer for *Aprostocetus* sp., 4.4 µl of the reverse primer, and 41.2 µl of ddH2O). PCR cycling conditions comprised of an initial denaturation at 94 °C for 3 minutes; followed by 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 50 seconds, and the final extension at 72 °C for 5 minutes.

Serial dilutions of DNA extracted from specimens of each RCS parasitoid species were prepared and used in multiplex PCR assay to determine primer sensitivity to lowest DNA concentration. The stock concentration of each parasitoid sample was: 2.89 ng/µl for *B. depressa*, 6.0 ng/µl for *N. japonicus*, 4.5 ng/µl for *Aprostocetus* sp., and 4.8 ng/µl for *A. lasallei*. Stock solution was added or diluted so each 25 µl reaction contained 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0 ng (negative control) of parasitoid DNA. Results are viewable in Suppl. material 3.

Gel electrophoresis

PCR products were visualized on 2.0% TAE agarose gels, made with TopVision Agarose Tablets (Thermo Fisher Scientific, Waltham, MA, USA), and stained with 1X SYBR™ Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) to confirm the success of PCR amplification.

Initial multiplex PCR test using actual RCS samples

The first test of the multiplex PCR with field-collected samples was conducted using DNA extracted from possibly parasitized RCS collected from the MRD in October 2022. Depending on the size of the immature and adult scales, we included multiple individuals within a sample, increasing the potential amount of parasitoid DNA within and making successful detection more likely. The sample set included 20 1st instar nymphs, 10 2nd instar nymphs, five 2nd instar nymphs, five 3rd instar nymphs, three 3rd instar nymphs, a single 3rd instar nymph, three 4th instar nymphs, two 4th instar nymphs, a single 4th instar nymph, three adult female scales, two adult female scales, a single adult female scale, and three male scales. Additionally, we included five positive controls, which consisted of DNA from *B. depressa*, *N. japonicus*, *Aprostocetus* sp., *A. lasallei*, all four wasps, and a negative control. Singleplex PCR was then performed on the same samples to confirm the results from the multiplex.

Life stage and seasonality parasitism frequency

Multiplex PCR was conducted using DNA extracted from individual scales collected at different times of the year to identify whether the frequency of parasitism by each species was variable by life stage and season. 100 1st instar nymphs, 20 2nd instar nymphs, 35 3rd instar nymphs, 35 4th instar nymphs, and 15 adult females were used from the October 2022 collection date. Only the 1st instar nymphs were pooled into the same sample, due to their small size, and they were never used in later dates due to the failure to detect any parasitoid DNA from them. 20 2nd instar nymphs, 30 3rd instar nymphs, 26 4th instar nymphs, and 14 adult females were used from the May 2023 collection date and 19 2nd instar nymphs, 16 3rd instar nymphs, 22 4th instar nymphs, and 20 adult females were used for August 2023.

Statistical analysis

The Pearson chi-square test was performed using JMP Pro 18.0.1 (SAS Institute, Cary, NC) to check whether observed species and multiparasitism frequencies from the PCR multiplex varied by seasonality. We tested the null hypothesis that the observed species frequencies and multiparasitism frequency were the same for all three sampling dates versus the alternative hypothesis that the frequencies were different for at least one sampling date.

Results

Primers for *Aprostocetus* sp.

Two potential forward primers for *Aprostocetus* sp. were designed and developed for use in multiplex PCR with the previously designed RCS parasitoid primers. As shown in Fig. 3, when the Aprostocetus_F2 primer was used (lanes 2 and 4) with the other encyrtid primers, DNA from all the four RCS parasitoids were produced. Even though the Aprostocetus_F1 primer properly amplified DNA of *Aprostocetus* sp. in multiplex PCR, only DNA of *N. japonicus* was amplified of the other species screened for, and a non-target band was produced around 600 bp (Fig. 3). Based on this result, the Aprostocetus_F2 primer was used for all subsequent PCR assays. Sequences were obtained for the PCR products of Aprostocetus_F2 with Encyrtid reverse primer and uploaded to GenBank (accession number PQ376138). The 267 bp sequence (after trimming the primers) was a 100% match with an internal section of the 582 bp 28S region we sequenced previously from *Aprostocetus* sp. (accession number PQ376137).

Initial multiplex PCR test using actual RCS samples

In the initial multiplex PCR test, wasp DNA was found from 2nd instar nymphs to adult females, but not from the 1st instars and males. Among four wasp species, *A. lasallei* DNA was detected in the widest range of life stages, from 2nd nymphs to adult female scales. DNA of *B. depressa* was detected from 2nd to 4th instar nymphs, but not from adult females. A DNA band for *N. japonicus* was produced from 3rd and 4th nymphal scales, and a DNA band for *Aprostocetus* sp. was only visualized from adult female scales (Fig. 4). To validate multiplex PCR results, singleplex

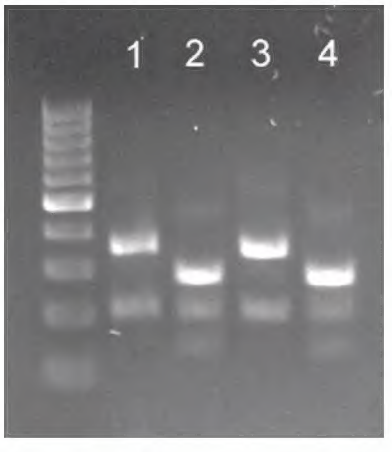

Figure 3. Aprostocetus_F1 primer was used for lanes 1 and 3, and Aprostocetus_F2 primer was used for lanes 2 and 4. 1 µl of DNA from each wasp was used for lanes 1 and 3, and 2 µl of DNA from each wasp was used for lanes 2 and 4. When Aprostocetus_F2 primer was used, DNA bands of all four wasps were visualized without non-target bands (lanes 2 and 4). The 155 bp band represents *B. depressa*, the 221 bp band denotes *N. japonicus*, the 375 bp (lanes 1 and 3) and the 303 bp band (lanes 2 and 4) indicates *Aprostocetus* sp., and the 495 bp band represents *A. lasallei*. With primer Aprostocetus_F1 a non-target band was produced at about 600 bp and the 155 and 495 bp bands were not amplified (lanes 1 and 3).

Figure 4. Wasp DNA detection results from RCS. 1 1st instar nymphs (n = 20) 2 2nd instar nymphs (n = 10) 3 2nd instar nymphs (n = 5) 4 3rd instar nymphs (n = 5) 5 3rd instar nymphs (n = 3) 6 3rd instar nymph (n = 1) 7 4th instar nymphs (n = 3) 8 4th instar nymphs (n = 2) 9 4th instar nymph (n = 1) 10 adult female scales (n = 3) 11 adult female scales (n = 2) 12 adult female scale (n = 1) 13 male scales (n = 3) 14 DNA of *B. depressa* 15 DNA of *N. japonicus* 16 DNA of *Aprostocetus* sp. 17 DNA of *A. lasallei*; 18 mixture of DNA from all four wasps 19 negative control. The 155 bp band represents *B. depressa*, the 221 bp band denotes *N. japonicus*, the 303 bp band indicates *Aprostocetus* sp., and the 495 bp band represents *A. lasallei*.

PCR for each wasp species was performed with the same RCS samples used in the initial multiplex PCR test. As shown in Fig. 5, all multiplex results were consistent with singleplex results.

Life stage and seasonality parasitism frequency

Individuals from each life stage across three collection dates were analyzed using the multiplex assay to confirm the observations made in the previous section and obtain parasitism frequencies for each life stage. Parasitoid DNA was never detected from samples extracted from the over 100 1st instar nymphs in October 2022 so was not repeated in the later dates. The earliest detection of *A. lasallei* occurred during the 2nd instar in all collection dates. *Boucekiella depressa* was detected as early as the 2nd instar for the May 2023 collection, but later in the 3rd instar during other dates. The earliest detection of *N. japonicus* occurred from the October 2022 collection during the 3rd instar but was only found in May 2023 from mature scales and only one 4th instar scale was parasitized by the species for the entire August 2023 collection. Only one scale was parasitized by *Aprostocetus* sp. prior to the 4th instar (Table 3). The observation frequencies of *B. depressa*, *N. japonicus*, and *Aprostocetus* sp. were not significantly different across the three sampling dates ($\chi^2(2, 274) = 2.48$, $p = 0.2891$, $\chi^2(2, 274) = 3.81$, $p = 0.1490$, $\chi^2(2, 274) = 0.04$, $p = 0.9796$, respectively) but the frequency of *A. lasallei* parasitism was higher in the October 2022 collection ($\chi^2(2, 274) = 9.47$, $p = 0.0088$).

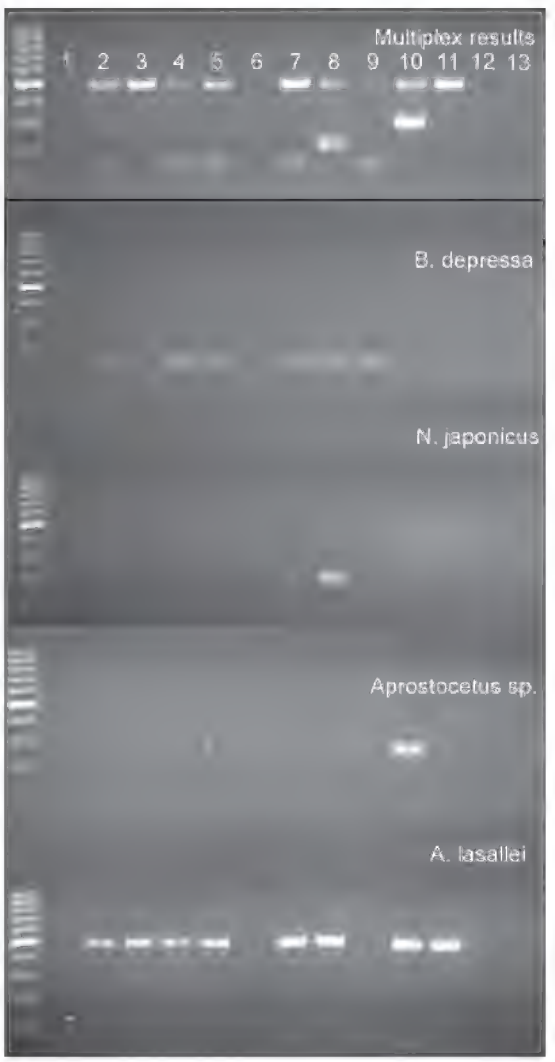

Figure 5. Wasp DNA detection results from RCS using multiplex PCR and comparison with singleplex PCR results. Multiplex PCR results are consistent with singleplex PCR results indicating all results are true positives: 1 1st instar nymphs (n = 20) 2 2nd instar nymphs (n = 10) 3 2nd instar nymphs (n = 5) 4 3rd instar nymphs (n = 5) 5 3rd instar nymphs (n = 3) 6 3rd instar nymph (n = 1) 7 4th instar nymphs (n = 3) 8 4th instar nymphs (n = 2) 9 4th instar nymph (n = 1) 10 adult female scales (n = 3) 11 adult female scales (n = 2) 12 adult female scale (n = 1) 13 male scales (n = 3). The 155 bp band represents *B. depressa*, the 221 bp band denotes *N. japonicus*, the 303 bp band indicates *Aprostocetus* sp., and the 495 bp band represents *A. lasallei*.

Multiparasitism was detected in 27 of the 98 parasitized scales (27.5%) across all three sampling dates. The frequency of multiparasitism was not affected by seasonality of collection χ^2 (2, 274) = 3.14, p = 0.2080. Of all multiparasitism events, the combination of *B. depressa* and *A. lasallei* was by far the most common, occurring 21 times (77.8%), *N. japonicus* and *A. lasallei* occurred three times (11.1%), and a single occurrence each of: *N. japonicus* and *Aprostocetus* sp., *B. depressa* and *Aprostocetus* sp., and a triplet of *B. depressa*, *A. lasallei*, and *N. japonicus* was detected.

Discussion

The newly developed forward primers successfully amplified DNA of *Aprostocetus* sp. extracted from mature wasps and larvae present in parasitized hosts. However, only the Aprostocetus_F2 primer interacted favorably when included in the multiplex to

Table 3. Frequency of parasitism (%) for each life stage by *B. depressa*, *N. japonicus*, *Aprostocetus* sp., and *A. lasallei* on three sampling dates as detected by the presence of bands during gel electrophoresis of PCR products.

Life Stage (n)	*B. depressa*	*N. japonicus*	*Aprostocetus* sp.	*A. lasallei*	Overall parasitism level (%)
October 2022					
1st (100)	0	0	0	0	0
2nd (20)	0	0	0	16.0	35.0
3rd (35)	11.4	8.6	0	42.9	45.7
4th (35)	17.1	11.4	5.7	51.4	62.9
Mature (15)	0	6.7	6.7	6.7	20.0
May 2023					
2nd (20)	15	0	0	45.0	50.0
3rd (30)	16.7	0	3.3	20.0	23.3
4th (26)	7.7	0	7.7	23.1	44.4
Mature (14)	7.1	28.6	0	14.3	42.9
August 2023					
2nd (19)	0	0	0	10.5	10.5
3rd (16)	6.3	0	0	56.3	56.3
4th (22)	13.6	4.5	9.1	13.6	27.3
Mature (20)	0	0	0	5.0	5.0

produce bands for all four wasp species (Fig. 3). The updated multiplex allows for the accurate detection and identification of member species in the RCS parasitoid complex, including larvae developing within the host. However, due to differences in annealing temperature by the constituent forward primers, DNA of certain species is amplified at greater intensities than others, leading to brighter bands for some and fainter for others. Forward primer concentrations have been adjusted to account for this, but further optimization still needs to be performed. We envision using the multiplex as a research tool to answer questions of biology and ecology for the RCS system, two of which we attempt to answer in this study: seasonal differences in parasitism and species frequency and the attack window for each parasitoid species.

The frequency for *B. depressa*, *N. japonicus*, and *Aprostocetus* sp. did not change seasonally, however *A. lasallei* was much more frequent in the October 2022 collection date. The frequency of multiparasitism did not vary by season. Multiparasitism was detected much more frequently with the molecular method than that reported in the native range by Cortez et al. 2022 (27% vs ~15%), which made use of rearing technique and was close to the 25% frequency found by Pruett et al. 2025 in their use of the original multiplex. *Astymachus lasallei* and *B. depressa* were found to be the most frequent multiparasitism combination in both our study, Cortez et al. 2022; Pruett et al. 2025. *Boucekiella depressa* has been reported as a primary parasitoid for other scale hosts as well as being a hyperparasitoid which may explain the frequency with which it was detected with other species and complicates its role in the system (Xia et al. 1993; Herting et al. 1997; Noyes 2019; Cortez et al. 2022). Our methodology does not allow for the identification of the exact physical location within the host the amplified DNA is contained, but the

detection of *B. depressa* present with no other species in several specimens indicates if it is a hyperparasitoid, then, hyperparasitism is likely facultative.

Our results show that not only does oviposition occur much earlier in host development than reported, but each species in the complex attacks in its own window of host development. We found that *A. lasallei* oviposits the earliest, during the second instar, *N. japonicus* and *B. depressa* arrive during the 3rd instar at earliest, finally *Aprostocetus* sp. is the last to oviposit, having mostly only been detected in 4th instar nymphs (with the exception of a single 3rd instar nymph) and fully mature female scales. Parasitoid DNA was never detected in 1st instar nymphs or mature males. From rearing techniques, we observed all adult wasps, regardless of species or multiparasitism, emerged simultaneously from adult-sized hosts and so detections at later host stages are most likely developing larvae that were oviposited earlier. The difference in oviposition timing and simultaneous adult emergence leads to the conclusion that there is also a variance in development speed between the parasitoids. *Astymachus lasallei*, *B. depressa*, and *N. japonicus* may then be qualified as koinobionts (a parasitoid that allows its host to continue development before killing it) (Harvey 2005) as the parasitized RCS nymph is able to undergo one or two additional instars before being completely consumed. While we observed no further development by the scale between oviposition by *Aprostocetus* sp. and complete consumption by the larvae, categorizing it as an idiobiont (Harvey 2005).

The temporal partitioning of resources, in this case by host life stage, is a method by which competition among species may be reduced and coexistence maintained. Previous work on intrinsic competition among parasitoids found that there is a priority advantage for ovipositing first. The typically inferior competitors had a head start on development and thus were larger and more likely to survive competition with the later ovipositing superior competitor (Hood et al. 2021). That metaanalysis mostly looked at solitary parasitoid larvae which typically have large mandibles to kill and consume rivals (Harvey et al. 2009). However, larvae of gregarious species, like those in the RCS system, lack these weapons and may not be capable of killing heterospecifics (Godwin and Odell 1984). Instead, gregarious parasitoids undergo scramble competition wherein all competitors are attempting to consume as much of a limited resource as possible, which favors those able to develop and consume resources the quickest (Wylie 1971, Harvey et al. 2013). Long-term coexistence among RCS parasitoids might be maintained by *A. lasallei* arriving earliest in host development but being a poor competitor and *Aprostocetus* sp. being a strong competitor but arriving last. *Neastymachus japonicus* and *B. depressa* populations likely represent an intermediate state between the two extremes and maintain coexistence by being just enough better competitor than *A. lasallei* and arriving just sooner than *Aprostocetus* sp. Future research should focus on determining how these parasitoids can discern nymphs of the appropriate life stage for oviposition when scales are typically covered by the leaf sheathe of their host.

Lastly, we attempt to explain a possible theory for why multiparasitism in the system is frequent. A single parasitized scale has enough resources for multiple individuals of the same species to complete development. Despite the difference in oviposition

timing between the species, there is still overlap where larvae may still be developing inside the host while the late arrival is ovipositing eggs. Since the larvae of neither species possess weapons with which to kill rivals, scramble competition occurs for what host resources are left. Since individual larva only need to consume part of the host to complete development, there is opportunity for at least some members of both species to complete development, particularly if that later arriving species can complete its development faster than the first species (Klomp and Teerink 1978).

Conclusion

The newly designed PCR multiplex is capable of recognizing and amplifying DNA specific to the four parasitoid wasp species that attack RCS in its adventive range. The new multiplex has uses as a research tool for the identification of parasitoid larvae within their host and answering questions of biology and ecology for this unusual system. We were able to use the multiplex to identify temporal resource partitioning with respect to oviposition timing which may be a possible explanation for how coexistence is maintained. Successful multiparasitism possibly still occurs because host resources are plentiful enough to support the development of multiple larvae, and later ovipositing competitors complete development fast enough that at least some number of both species complete development.

Acknowledgements

We would like to thank Ian Knight and Keyla Pruett for initial primer design and development of the original multiplex. Additional thanks to Josh Snook, Logan Herbert, and Matt Berry for boat transportation and help collecting scales from field sites. We appreciate the efforts of the anonymous reviewers of this paper and their recommended changes. Funding for this project came from the USDA-APHIS Award Number AP22PPQS&T00C060 to Rodrigo Diaz. This material was made possible, in part, by a Cooperative Agreement from the United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS). It may not necessarily express APHIS' views. The findings and conclusions in this publication are those of the authors and should not be construed to represent any official USDA or U.S. Government determination or policy.

References

Abell, KJ, Duan JJ, Shrewsbury PM (2020) Determining optimal parasitoid release timing for the biological control of emerald ash borer (Coleoptera: Buprestidae). Florida Entomologist 102(4): 691–694. https://doi.org/10.1653/024.102.0403

Agusti N, Bourguet D, Spataro T, Delos M, Eychenne N, Folcher L, Arditi R (2005) Detection, identification and geographical distribution of European corn borer larval parasitoids using molecular markers. Molecular Ecology 14(10): 3267–3274. https://doi.org/10.1111/j.1365-294X.2005.02650.x

Brix H, Ye S, Laws EA, Sun D, Li G, Ding X, Yuan H, Zhao G, Wang J, Pei S (2014) Large-scale management of common reed, *Phragmites australis*, for paper production: A case study from the Liaohe Delta, China. Ecological Engineering 73: 760–769. https://doi.org/10.1016/j.ecoleng.2014.09.099

Campbell BC, Steffen-Campbell JD, Werren JH (1994) Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. Insect molecular biology 2(4): 225–237. https://doi.org/10.1111/j.1365-2583.1994.tb00142.x

Cortez Jr AO, Chu CL, Broadley HJ, Lo YS, Chen YC, Gates MW, Meyerson LA, Hoelmer KA, Gould JR, Hwang SY (2022) Exploratory surveys in Taiwan of the roseau cane scale *Nipponaclerda biwakoensis* Kuwana (Hemiptera: Aclerdidae) and its associated parasitoids. Journal of Applied Entomology 146(5): 596–606. https://doi.org/10.1111/jen.12982

Cronin JT, Johnston J, Diaz R. (2020) Multiple Potential Stressors and Dieback of *Phragmites australis* in the Mississippi River Delta, USA: Implications for Restoration. Wetlands. https://doi.org/10.1007/s13157-020-01356-8

Oligo Analysis Tool (2023) Eurofins Oligo Analysis Tool. https://eurofinsgenomics.com/en/resources/tools/oligo-analysis/ [Accessed on 15.05.2023]

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M (2007) Parasitoids, predators and PCR: the use of diagnostic molecular markers in biological control of Arthropods. Journal of Applied Entomology 131(4): 225–40. https://doi.org/10.1111/j.1439-0418.2007.01145.x

Godwin PA, Odell TM (1984) Laboratory study of competition between *Blepharipa pratensis* and *Parasetigena silvestris* (Diptera: Tachinidae) in *Lymantria dispar* (Lepidoptera: Lymantriidae). Environmental entomology 13(4): 1059–1063. https://doi.org/10.1093/ee/13.4.1059

Greenstone MH (2006) Molecular methods for assessing insect parasitism. Bulletin of entomological research 96(1): 1–3. https://doi.org/10.1079/BER2005402

Hancock JM, Tautz D, Dover GA (1988) Evolution of the secondary structures and compensatory mutations of the ribosomal RNAs of *Drosophila melanogaster*. Molecular Biology and Evolution 5(4): 393–414. https://doi.org/10.1093/oxfordjournals.molbev.a040501

Harvey JA (2005) Factors affecting the evolution of development strategies in parasitoid wasps: the importance of functional constraints and incorporating complexity. Entomologia experimentalis et applicata 117(1): 1–13. https://doi.org/10.1111/j.1570-7458.2005.00348.x

Harvey JA, Gols R, Strand MR (2009) Intrinsic competition and its effects on the survival and development of three species of endoparasitoid wasps. Entomologia Experimentalis et Applicata 130(3): 238–248. https://doi.org/10.1111/j.1570-7458.2008.00816.x

Harvey JA, Poelman EH, Tanaka T (2013) Intrinsic inter-and intraspecific competition in parasitoid wasps. Annual review of entomology 58(1): 333–351. https://doi.org/10.1146/annurev-ento-120811-153622

Herting B (1997) Section A. Host or Prey/Enemy. In: Hymenoptera. A catalogue of parasites and predators of terrestrial arthropods (Vol. 4). Commonwealth Agricultural Bureaux, Institute of Biological Control, 206 pp.

Hood GR, Blankinship D, Doellman MM, Feder JL (2021) Temporal resource partitioning mitigates interspecific competition and promotes coexistence among insect parasites. Biological Reviews 96(5): 1969–1988. https://doi.org/10.1111/brv.12735

JMP Pro 18.0.1 (1989–2023) JMP Pro®, Version 18.0.1. SAS Institute Inc., Cary, NC, 1989–2023.

Kaneko S (1995) Frequent successful multiparasitism by five parasitoids attacking the scale insect *Nipponaclerda biwakoensis*. Population Ecology 37(2): 225–228. https://doi.org/10.1007/BF02515824

Kaneko S (2004) Within-plant vertical distributions of the scale insect *Nipponaclerda biwakoensis* and its five parasitoids that exhibit frequent successful multiparasitism on the common reed. Entomological Science 7(4): 331–339. https://doi.org/10.1111/j.1479-8298.2004.00081.x

Kaneko S (2005a) Seasonal population changes of five parasitoids attacking the scale insect *Nipponaclerda biwakoensis* on the common reed, with special reference to predation by wintering birds. Entomological Science 8(4): 323–329. https://doi.org/10.1111/j.1479-8298.2005.00131.x

Kaneko S (2005b) Abundances of five parasitoids attacking the scale insect *Nipponaclerda biwakoensis* on morphologically changed reed shoots due to damage by a stem-boring caterpillar. Ecological research 20: 555–561. https://doi.org/10.1007/s11284-005-0068-3

Klomp H, Teerink BJ (1978) The elimination of supernumerary larvae of the gregarious egg-parasitoid *Trichogramma embryophagum* [Hym.: Trichogrammatidae] in eggs of the host *Ephestia kuehniella* [Lep.: Pyralidae]. Entomophaga 23(2): 153–159. https://doi.org/10.1007/BF02371721

Knight IA, Wilson BE, Gill M, Aviles L, Cronin JT, Nyman JA, Schneider SA, Diaz R (2018) Invasion of *Nipponaclerda biwakoensis* (Hemiptera: Aclerdidae) and *Phragmites australis* dieback in southern Louisiana, USA. Biological Invasions 20: 2739–2744. https://doi.org/10.1007/s10530-018-1749-5

Knight IA, Cronin JT, Gill M, Nyman JA, Wilson BE, Diaz R (2020) Investigating Plant Phenotype, Salinity, and Infestation by the Roseau Cane Scale as Factors in the Die-Back of *Phragmites australis* in the Mississippi River Delta, USA. Wetlands 40: 1327–1337. https://doi.org/10.1007/s13157-020-01307-3

Lopez LAA (2022) Ecology of the Roseau Cane Scale (*Nipponaclerda biwakoensis*, Hemiptera: Aclerdidae) in Coastal Louisiana. Master's Thesis, Louisiana State University, Louisiana. https://doi.org/10.31390/gradschool_theses.5202

McConnell HS (1953) A Classification of the Coccid Family Aclerdidae (Coccoidea, Homoptera). Bulletin Maryland Agriculture Experiment Station A75: 1–121.

Miller JC (1982) Life history of insect parasitoids involved in successful multiparasitism. Oecologia 54: 8–9. https://doi.org/10.1007/BF00541100

Noyes JS (2019) Universal Chalcidoidea Database. http://www.nhm.ac.uk/chalcidoids [Accessed on 07.08.2024]

Noyes JS and Higashiura, Y (2020) The species of *Astymachus* Howard (Hymenoptera: Encyrtidae), potentially important parasitoids of Aclerdidae (Hemiptera: Coccoidea) associated with grasses (Poaceae), with descriptions of three new species. Journal of Natural History 54(9–12): 665–679. https://doi.org/10.1080/00222933.2020.1747654

Pruett KN, Knight IA, Kang I, Sparks VL, Diaz R (2025) Species-specific PCR primers provide insights into spatial distributions and interspecific interactions of roseau cane scale parasitoids in Louisiana. Biocontrol Science and Technology. https://doi.org/10.1080/09583157.2025.2563728

Ramsey III EW, Rangoonwala A (2017) Mapping the change of *Phragmites australis* live biomass in the lower Mississippi River Delta marshes (No. 2017-1098). US Geological Survey. https://doi.org/10.3133/ofr20171098

Schneider SA, Broadley HJ, Andersen JC, Elkinton JS, Hwang SY, Liu C, Noriyuki S, Park JS, Dao HT, Lewis ML, Gould JR (2022) An invasive population of Roseau Cane Scale in the Mississippi River Delta, USA originated from northeastern China. Biological Invasions 24: 2735–2755. https://doi.org/10.1007/s10530-022-02809-3

Smith MA, Woodley NE, Janzen DH, Hallwachs W, Hebert PD (2006) DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). Proceedings of the National Academy of Sciences, 103(10): 3657–3662. https://doi.org/10.1073/pnas.0511318103

Smith MA, Rodriguez JJ, Whitfield JB, Deans AR, Janzen DH, Hallwachs W, Hebert PD (2008) Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. Proceedings of the National Academy of Sciences 105(34): 12359–12364. https://doi.org/10.1073/pnas.0805319105

Tilmon KJ, Danforth BN, Day WH, Hoffmann MP (2000) Determining parasitoid species composition in a host population: a molecular approach. Annals of the Entomological Society of America 93(3): 640–647. https://doi.org/10.1603/0013-8746(2000)093[0640:DPSCIA]2.0.CO;2

Traugott M, Zangerl P, Juen A, Schallhart N, Pfiffner L (2006) Detecting key parasitoids of lepidopteran pests by multiplex PCR. Biological Control 39(1): 39–46. https://doi.org/10.1016/j.biocontrol.2006.03.001

Wylie HG (1971) Observations on intraspecific larval competition in three hymenopterous parasites of fly puparia. The Canadian Entomologist 103(1): 137–142. https://doi.org/10.4039/Ent103137-1

Xia BC, Zhao YQ, Shen BY (1993) The ecological control of pests and diseases of reed in Shenyang beach. Journal of Plant Resources and Environment 2: 31–36.

Zhou QS, Xi YQ, Yu F, Zhang X, Li XJ, Liu CL, Niu ZQ, Zhu CD, Qiao GX, Zhang YZ (2014) Application of DNA barcoding to the identification of Hymenoptera parasitoids from the soybean aphid (*Aphis glycines*) in China. Insect Science 21(3): 363–373. https://doi.org/10.1111/1744-7917.12095

Supplementary material 1

Phragmites australis die-back in the Mississippi River Delta

Authors: Violet Sparks, Ilgoo Kang, Morgan Carrere, Hannah Broadley, Rodrigo Diaz

Data type: jpg

Explanation note: Die-back of *Phragmites australis* surrounded by healthy stands within the Mississippi River Delta, Louisiana, USA.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/jhr.98.150721.suppl1

Supplementary material 2

Oil and natural gas infrastructure is dispersed throughout the Mississippi River Delta

Authors: Violet Sparks, Morgan Carrere

Data type: jpg

Explanation note: An oil pumping station is surrounded by *Phragmites australis* within the Mississippi River Delta, Louisiana, USA.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/jhr.98.150721.suppl2

Supplementary material 3

Serial dilutions of stock parasitoid DNA for testing primer sensitivity

Authors: Violet Sparks, Ilgoo Kang, Morgan Carrere, Hannah Broadley, Rodrigo Diaz

Data type: png

Explanation note: Results of primer specificity testing. Serial dilutions were performed so each 25 µl reaction contained 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0 ng (negative control) of stock parasitoid DNA (lanes 1–8 from greatest to least DNA concentration).

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/jhr.98.150721.suppl3